A DISSERTATION FOR THE DEGREE OF MASTER

Comparison of Stress Level Affected by Training Methods Using Salivary Cortisol Measurement in Dogs

개에서 타액 코티솔 측정을 통한 훈련방법에 따른 스트레스 유발 수준의 비교

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김 소 희

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Comparison of Stress Level Affected by Training Methods Using Salivary Cortisol Measurement in Dogs

By
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A Dissertation submitted to
the Graduate School of Seoul National University in partial fulfillment
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in Animal Behavioral Medicine

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Abstract

The objective of this study was to investigate the effect of the reward-based training method and punishment-based training method among dog training methods through salivary cortisol concentration measurements. For canines to live among humans in a physically and healthy manner, socialization and basic obedience training are necessary. A number of training methods are available today. In this study, in order to investigate the differences in stress variation experienced by canines depending on the training method, the salivary cortisol level variations before (Sx-0) and after training sessions (Sx-1) were measured and compared. Dogs reward-based training facilities (test group R, n=10) and dogs in punishment-based training facilities (test group P, n=9) underwent 3 basic obedience training sessions (Sx, x=1, 2, 3), which included the commands "sit", "lie down", and "wait". Saliva samples were collected by inducing the dogs to hold the swabs in their cheek pouches for 1 minute. Analysis of the samples was carried out using a high sensitivity salimetrics kit. After measuring the average optical density (O.D.) of each saliva sample, the calculated salivary cortisol concentration value was converted to ㎍/㎗. Result from the comparison of cortisol concentration variation before and after each training session for each test group showed that for S1, the salivary cortisol concentration variation before and after the session for test groups R and P were 0.037±0.030 μg/dℓ and 0.145±0.045 μg/dℓ, respectively. The increase in cortisol concentration was significantly smaller for test group R and exhibited statistical significance ($p<0.05$). Similarly for S2, the salivary cortisol concentration variation before and after each training session for test groups R and P were -0.024±0.030 μg/dℓ and 0.132±0.102 μg/dℓ, respectively. The increase in cortisol concentration of test group R was significantly smaller ($p<0.1$). For S3, the salivary cortisol concentration variation before and after each training session for test groups R and P were -0.108±0.077 μg/dℓ and -0.011±0.019 μg/dℓ, respectively. Again, the smaller cortisol increase was seen in test group R, but it was not statistically significant. It was also found that in both groups, the degree of increase in salivary cortisol concentration reduced as each session repetition of the training was completed. In group R, the relationships between S1 and S2 as well as S1 and S3 were statistically significant ($p<0.1$). In group P, the relationships between S2 and S3 as well as S1 and S3 were statistically significant ($p<0.05$). Through this study, it was found that reward-based training methods resulted in substantially smaller stress increases after the training when compared to punishment-based training methods. Therefore, the results of this study suggest that reward-based training methods are more ethical and beneficial towards animal welfare. The finding of this study are expected to contribute to related future research.

Keywords: dog, salivary cortisol, training method, stress
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1. Introduction

In the U.S., 40% of all households have at least one pet dog and the total number of dogs reached approximately 72 million (AVMA, 2007). 90% of these pet dogs have at least one behavioral problem (Vacalopoulos and Anderson, 1993). Representative behavioral problems include aggression, excessive barking, destructive behavior, and house soiling (Beaver, 2009). These problems are major causes of social issues, such as animal abuse, disownment, abandonment, and euthanasia (Beaver, 2009; Blackwell *et al.*, 2008; Patronek *et al.*, 1995; Salman *et al.*, 1998; Wells and Hepper, 2000). Annually, 3−4 million dogs are abandoned at shelters; 30−46% of these dogs exhibit a mix of behavioral problems, while 25% of the dogs have behavioral and health−related problems, making them unfit for adoption. Half of the dogs left at shelters are euthanized and 18.8% of the dogs adopted are disowned (Luescher and Tyson, 2009; Beaver, 2009; Wells and Hepper, 2000; Patronek *et al.*, 1995) Thus, it can be considered that behavioral issues are directly related with the welfare and survival of dogs. In Korea, 21.2% of all households have pet dogs (National Veterinary Research and Quarantine Service, 2007), and approximately 100 thousand abandoned animal cases are reported each year (Yun *et al.*, 2014). There were no other related studies and it was predicted that the situation in the U.S. would not be drastically different.

Training is necessary to solve the behavioral problems of dogs which leads to social issues. As basic obedience training is necessary for working dogs that independently perform tasks in various environments, yet must also adhere to the control of their handlers at all times (NASAR, 1999), domesticated dogs who are pets that live closely with people require basic training and socialization training (Dunbar, 2004; Serpell, 1996). Training not only enhances obedience and prevents behavioral problems in advance, but can also rectify behavioral issues, potentially preventing problems such as abandonment and the administration of euthanasia (Arhant *et al.*, 2010; Greenebauma, 2010). A study by Jagoe and Serpell (1996) demonstrates that dog training causes specific behavioral problems, however, it appears that this was caused by the training method used (Arhanta *et al.*, 2010; Hiby *et al.*, 2004).

Most methods for dog training are based on operant conditioning, a training principle systematized by Skinner. Operant conditioning gives or takes away something as a reward for some action and identifies 4 categories depending on whether that action is increased or decreased. These categories are positive reinforcement (+R) or increasing a behavior by giving something, negative punishment (−P) or reducing a behavior by taking away something, negative reinforcement (−R) or increasing a behavior by taking away or reducing something, and positive punishment (+P) or reducing a behavior by giving something.

These 4 categories then can be divided into two types (Arhant *et al.*, 2010; Hiby *et al.*, 2004). According to Arhant *et al.* (2010), reward-based methods generally involves providing positive reinforcement (+R) to the dog in a pleasant manner when the dog shows a desirable behavior while negative punishment (−P) is provided to stops the positive reinforcement when undesirable behavior is exhibited. However, this does not entail an actual aversive interaction. In contrast, punishment-based methods involve the unpleasant interaction between the dog and its caretaker. When the dog shows an undesirable behavior, positive punishment (+P) is given to the dog such as surprising the dog or even physical pain, while negative reinforcement (−R) is provided when the dog shows desirable behavior as a form of reward.

The relative advantages and disadvantages of these two training techniques are still under debate, but relevant comparative research has recently begun (Rooney and Cowan, 2011). Rooney and Cowan (2011) videotaped and analyzed owners of domesticated dogs of varying breeds training their dogs to perform a certain task. It was found that the dogs of owners that mainly employed punishment-based methods interacted and played less with people they were not familiar with, while the dogs of owners that mainly employed reinforcement-based methods were playful and were better at learning new behaviors. Since the frequency of playtimes can be an index for measuring stress (Hováth *et al.*, 2008) so this result implies that the dogs trained using reinforcement-based methods experience lower levels of stress.

In addition, Deldalle and Gaunet (2014) visited regular pet dog training schools who used +R and −R methods and performed behavioral analyses of the dog owners and their dogs, who participated in their training session. The dogs were diverse breeds and sexes. 65% of the dogs at the school that mainly used punishment-based methods showed at least one form of stress related behavior (mouth licking, yawning, scratching, sniffing, shivering, whining, low posture or avoidance behavior [gaze direction, body incline]), while only 8% of the dogs at the school that used mainly reinforcement-based training methods showed such behavior. Thus, the latter training method was found to be less stressful for the dogs and closer to providing welfare for the dogs.

Lastly, Herron *et al.* (2009) studied pet dog owners and performed behavioral analyses to find that at least 25% of the dogs trained using undesirable actions, including hitting, kicking, growling at the dog, physical force, alpha roll, and grab and shake showed aggressive responses. However, the dogs that received reinforcement-based methods rarely showed such aggressiveness.

The cortisol level is a suitable stress index for dogs

(Beerda, *et al.*, 1999a; Coppola *et al.*, 2006). The main biological measurement techniques that have been used to measure the effect of various stressful situations on domestic dogs have been blood cortisol and heart rate monitoring (Clark *et al.*, 1997; Dreschel and Granger, 2005; Hennessy *et al.*, 1998); however, recently, more studies using non-invasive methods such as salivary cortisol collection (Beerda *et al.*, 1996; Coppola *et al.*, 2006; Dreschel and Granger, 2009), spurred by an interest in the welfare and ethics of dogs, has gained momentum (Beerda *et al.*, 1998; Haverbeke *et al.*, 2008a). The method of measuring stress hormones by collecting blood is an invasive procedure that requires professional skill, while saliva sample collection is non-invasive and effectively reflects the blood hormone levels (Beerda *et al.*, 1996; Coppola *et al.*, 2006; Vincent and Michell, 1992). They are also easy to carry out in non-laboratory and clinic environments (Dreschel and Granger, 2009). Due to these advantages, the salivary method for measurement of cortisol concentration is widely used to measure the stress levels of dogs (Kobelt *et al.*, 2003).

Based on the results of the preceding literature review, it was hypothesized that the amount of stress experienced by dogs differs depending on whether punishment-based or reinforcement-based training methods were employed. Two main experiments that compared the two contrasting training methods were carried out. They examined the difference in the salivary cortisol concentrations before and after presenting the stressor. This study referenced the experiment design and methodology of previous studies.

The objective of this study was to investigate differences in stress levels induced by different training methods. We hypothesize that the reward−based training method is less stress−inducing than punishment−based training. For the reward−based training method and punishment−based training method, experimental groups R (n=10) and P (n=9) were prepared, respectively. Both groups underwent 3 basic obedience training sessions. Sx−0 and Sx−1 salivary cortisol concentration measurements were collected and variations in the levels compared at each session. It was predicted that the results of this study would show which training method causes less stress.

This study was carried out under the approval (SNU−121109−1) of the Seoul National University Institutional Animal Care and Use Committees.

2. Materials and Methods

2.1. Experiment Animals

Nineteen physically healthy dogs were studied. All the dogs were pet dogs of regular households and entrusted to the training school. Training schools using reward-based and punishment-based training methods were contacted separately for this study. Since previous studies show that the sex, breed, and age of the dogs had no impact on the salivary cortisol concentration value (Coppola *et al.*, 2006; Sandri *et al.*, 2015), the sex of the dogs was not considered for this study. Only medium and large size breeds related to the sporting and herding groups (AKC, 2016) were considered for this study. Two training facilities were selected; one used the reward-based training method, while the other used the punishment-based method. These facilities were selected because they had structures with separate kennels and yards so that the dogs could not see the other dogs receiving the training.

The experiment was comprised of test group R (n=10), which used the reward-based method, and test group P (n=9), which used the punishment-based method. The breeds included in test group R were 1 Belgian sheep dog (B.S.), 3 border collies (B.C.), 2 golden retrievers (G.R.), 2 labrador retrievers (L.R.), and 2 Welsh corgis (W.C.). Their average age was

5.0±0.47 (years, mean±S.E.) and the sex ratio was 5 female dogs to 5 male dogs. The breeds of test group P were 4 German sheep dogs (G.S.), 4 golden retrievers (G.S.) and 1 labrador retriever (L.R.). Their average age was 3.0±0.53 (years, mean±S.E.) and the sex ratio was 4 female dogs to 5 male dogs (Table 1). No dogs had been neutered.

Additional selection criteria that were considered included the following: medication taken by the experimental animals within the month prior to entering the training facility, and at the start of the study and lack of diseases. Dogs in heat, pregnant female dogs (Dreschel and Granger, 2009), dogs less than one year of age and susceptible to being affected by the circadian rhythm, and geriatric dogs greater than 8 years of age were excluded (Palazzolo and Quadri, 1987). When a dog is introduced into a new environment, activation of the hypothalamic −pituitary−adrenocortical (HPA) axis, a major component of the brain neuroendocrine system, has been found to improve (Beerda *et al.*, 1997). Therefore, in order to ensure that the dogs in the dogs in the experiment were in the most stable condition, only dogs that had been at entered the training school for at least 5 days were considered for this study. In addition, in order to familiarize them with the training location prior to the start of the experiment, the dogs were taken on walks to the field at least twice a day before commencing experimental activities (Beerda *et al.*, 1997). Based on the results of the study by

Horváth *et al.* (2008), which showed that dogs should be in their kennels for at least 30 minutes before an experimental activity, the dogs in this study were not allowed to be physically active 1 hour prior to the training sessions and left in their kennels with minimal stimulation (Horváth *et al.*, 2008). Food and drinking water were limited an 1 hour before the training (Dreschel and Granger, 2009), however, during the experimental period, the dogs were provided with unscheduled meals and drinking water. Furthermore, in order to ensure the most comparable experimental environments as possible, two training schools were selected based on having separated kennels and fields and a structure where the other dogs could not see the training or saliva collection.

Table 1. Breeds, ages, and sexes of the dogs used in the experiment

Training method	Animal No.	Breed	Age	Sex
Group R	1	L.R.	3	F
	2	G.R.	7	F
	3	B.C.	4	F
	4	W.C.	5	M
	5	B.C.	7	F
	6	B.C.	6	M
	8	L.R.	6	M
	9	W.C.	5	M
	10	G.R.	6	M
Group P	1	L.R.	4	F
	2	G.S.	2	M
	3	G.R.	2	M
	4	G.S.	2	M
	5	G.S.	2	F
	6	G.R.	6	F
	7	G.R.	4	F
	8	G.R.	4	M
	9	G.S.	1	M

Group R, Reward−based method; Group P, Punishment−based method; G.R., golden retriever; L.R., labrador retriever; G.S., German shepherd Dog; W.C., Welsh corgi; B.S., Belgian sheepdog; B.C., border collie; F, female; M, male

2.2. Training Methods

Test group R underwent the experiment at the training facility employing the reward-based training method and test group P underwent the experiment at the training facility employing the punishment-based training method. So, for test group R, the dogs were made to focus continuously through +R, providing compliments and rewards when the dogs exhibit the behavior desired, placing the emphasis of the training on inducing the desired behavior. −P is used when undesired behavior is displayed by eliminating pleasant interactions, for example, showing apathy. +P such as using a choke chain, physical force, shouting, or establishing an imposing atmosphere. On the other hand, for test group P, +P was used including using a leash, pulling, dragging, pressing with hands, glaring, shouting, and overpowering. When the desired behavior was exhibited, −R disable the stimulation was used. In accordance with the protocols of Dreschel and Granger (2009) and Salimetrics, Inc., where the dogs were not fed 20 minutes before the sample collection, food rewards were not considered for either training methods.

Generally, in basic obedience training, the commands "sit", "down", "wait", "heel", and "come" are included (Alexander *et al.*, 2011; Seksel *et al.*, 1999), while the two test groups were trained with only the commands "sit", "down", and "wait".

Based on previous results, which showed that training sessions that were equal to or less 10 minutes in duration and repeated frequently generates the most favorable results (Pryor, 1999), a total of 3 training sessions (1 session per day for 3 consecutive days) were carried out for each dog.

The training was conducted within each training facility, and the training session and saliva collection were always done at the same locations where the other dogs could not see. For each training facility, 1 expert trainer with more than 5 years of experience conducted all the training exercises from S1 to S3. The trainer had previous knowledge about the experiment investigating the effect of training on the endocrine system of dogs.

2.3. Saliva Sampling

Children' s Swab (SCS, Salimetrics, PA, USA) was used for collecting the dog saliva. The collection and treatment method of the saliva samples followed the protocol of Salimetrics, Inc. (2011, 2nd edition, Salimetrics, LLC). Saliva collection was performed as soon as the dogs were taken out off the kennel (Sx−0, x=1, 2, 3) and again 1 minute after each 10 minute training session (Sx−1, x=1, 2, 3).

Because Castillo *et al.* (2009) stated "the dog may display significant individual circadian variability," all sample collections were performed once a day between 1 p.m. and 4 p.m. This was done for 3 consecutive days in October (October 3, 4, 5 for group R and October 8, 9, 10 for group P), so that the experimentation and saliva sample collection were all carried out during the same time period in as little amount of time as possible.

According to the study by Dreschel and Granger (2009), if the sample collection method was educated, then there was no correlation between the collecting personnel and the salivary cortisol concentration and the amount collected. Therefore, in order to minimize the possible effect of a stranger (experimenter) on the dogs, the trainer responsible for the training of each test group also collected the saliva before and after the sessions. For the saliva collection, half of the SCS packaging was peeled, one end of the swab was held, and the other end was placed under the tongue or the side cheek. If necessary, the dog was instructed to close its mouth and the dog' s cheek pouch was massaged. Swabs that were contaminated with blood or dropped on the ground were not used as samples.

According to Salimetrics, Inc., user manual (2011, 2nd edition, Salimetrics, LLC) and the study by Dreschel and Granger (2009), a 25 ㎕ sample of saliva is necessary. Since insufficient saliva amount could lead to inappropriate results (Harmon *et al.*, 2007), the saliva collection was extended to 2 rounds, 30 seconds each. This was based on literature that shows when sampling is carried out for 1 minute (Dreschel and Granger, 2009), but the collected amount is not 0.2 ㎖, saliva collection can be done for up to 4 minutes without affecting the concentration handling (Kobelt *et al.*, 2003). Methods to induce salivary secretion in an effort to prevent reduction of the efficacy of ELISA were not used (Ligout *et al.*, 2009). When a sufficient amount of saliva was absorbed into the swab, the swab was cut, with sterilized scissors, at a point where it had stopped absorbing saliva. The cut swab was placed in a 5 ㎖ needleless syringe (Greenject-5, Doo won meditech Co., Ltd.) then extracted and placed a swab storage tube (SST, Salimetrics, PA, USA). The extracted saliva was labeled then immediately stored in a cooler with an ice pack followed by storage in a -20˚C freezer during the day of collection until the day of analysis (Harley *et al.*, 1998).

2.4. Salivary Cortisol Measurement

When the saliva sample is frozen, the mucin precipitates so it was melted in room temperature on the day of the experimentation, shaken well, and centrifuged for 15 minutes at approximately 3000 rpm. Only the supernatant, without the precipitation on the bottom, was used. The highly sensitive (from 0.003 ㎍/㎗ to 3.0 ㎍/㎗) Salivary Cortisol Enzyme Immunoassay kit (Salimetrics, PA, USA) was used for the salivary cortisol analysis. The procedure from the Salimetrics, Inc., protocol was followed (Rev. March 2011, Salimetrics). After measuring the absorbance of each sample, the salivary cortisol concentration was converted to ㎍/㎗.

2.5. Statistical Analysis

The converted salivary cortisol concentration value for each sample was statistically analyzed. SPSS (SAS 9.4) was used for the data analysis and statistical treatment. A *t*−test was performed to verify differences between the Sx−0 values of both groups and the basal values. Corresponding analysis was carried out in order to determine if there was variation between the Sx−0 and Sx−1 values for each group. An independent *t*−test was carried out to determine if the difference in the variation of cortisol level before and after each session, depending on the training method, was a statistically significant. A test for homoscedasticity for each group was conducted before carrying out the independent *t*−test. The result showed that the significant probabilities for S2 and S3 were less than the 5% significance level, thus the distribution of the stress variation depending on the training method did not have equal variance. Thus, an independent *t*−test assuming homoscedasticity was performed for S1, while independent *t*−tests assuming heteroscedasticity was performed for S2 and S3. An ANOVA test was conducted to check for salivary cortisol concentration variations as the training session for each group was carried out. A *t*-test was performed to determine the difference between the two sessions.

3. Results

There were 120 samples collected from 19 dogs. Cases when the dog swallowed the swab, blood contaminated the sample, the sample fell on the ground, or there was no matching pair were excluded and a final total of 110 samples were used. In many previous studies, beef flavor or citric acid stained swabs or having the dogs smell food were methods used to collect the saliva of dogs. However, in this study, sufficient amounts were collected without the use of these additional methods.

The results of the *t*−tests, performed determine the difference between the salivary cortisol concentration before the experiment (Sx−0, x=1, 2, 3) for the two test groups were as follows: the S1−0 values for group R and group P were 0.223±0.029 ㎍/dℓ and 0.272±0.066 ㎍/dℓ, the S2−0 values were 0.354±0.077 ㎍/dℓ and 0.375±0.065 ㎍/dℓ, and the S3−0 values were 0.370±0.066 ㎍/dℓ and 0.434±0.079 ㎍/dℓ. All the values showed no statistical significance ($p>0.1$). Finally, the averages for Sx−0 (x=1, 2, 3) of each group was as follows: 0.321±0.037 ㎍/dℓ and 0.363±0.041 ㎍/dℓ, respectively. This showed that the basal levels before experimentation for both groups were the same ($p>0.1$, Figure 1).

In S1, the salivary cortisol concentration average values for S1−0 and S1−1 for test group R were 0.223±0.029 ㎍/dℓ and

0.260±0.038 ㎍/㎗ respectively. The average values for S1-0 and S1-1 for test group P were 0.272±0.066 ㎍/㎗ and 0.417±0.099 ㎍/㎗, respectively. In S2, the average values for S2-0 and S2-1 for test group R were 0.354±0.077 ㎍/㎗, and 0.330±0.072 ㎍/㎗, respectively, while the values for S2-0 and S2-1 for test group P were 0.375±0.065 ㎍/㎗ and 0.507±0.160 ㎍/㎗, respectively. Lastly in S3, the values for S3-0 and S3-1 for test group R were 0.370±0.066 ㎍/㎗ and 0.262±0.035 ㎍/㎗, respectively, while the obtained values for S3-0 and S3-1 for test group P were 0.434±0.079 ㎍/㎗ and 0.423±0.077 ㎍/㎗, respectively (Figure 2). Similarly, the salivary cortisol concentrations before and after each session for each group showed individual trends of no statistical significance. However, the difference in the degree of increase between the groups was statistically significant. In other words, looking at the increase between the S1-0 and S1-1 for each group, the degree of salivary cortisol concentration increase for group R was significantly less than that of group P ($p<0.05$). Based on these results, the variations from before (Sx-0) and after each session (Sx-1) for the two groups were calculated (Figure 3). For S1, the salivary cortisol concentration variations for test groups R and P were 0.037±0.030 ㎍/㎗ and 0.145±0.045 ㎍/㎗, respectively. The cortisol increase of test group R was significantly smaller ($p<0.05$). Therefore, the induced stress from training when using the reward-based method was less than when using the punishment-based method. For S2, the variation in salivary cortisol concentration for test groups R and P were -0.024±0.030 ㎍/㎗ and 0.132±0.102 ㎍/㎗ respectively. The degree of cortisol increase for test group R also was significantly smaller than group P ($p<0.1$). Thus, at S2, the induced stress from training for the reward-based training method was also lower than the punishment-based training method. For S3, the stress after training decreased for both groups and the salivary cortisol concentration variations for group R and group P were −0.108±0.077 ㎍/㎗ and -0.011±0.019 ㎍/㎗, respectively. This was similar to the change in salivary cortisol concentration where group R had less of an increase than group P, but it was not statistically significant.

Groups R and P showed a clear trend of a decreasing degree in the increase od salivary cortisol concentration when compared before and after experimentation, as the training session progressed from S1 to S3. While all the sessions were not statistical significant, the difference between two sessions was significant. For group R, the increase for S2 was less than the increase for S1, and the increase for S3 was less than the increase for S1. Each showed statistical significance ($p<0.1$, Figure 4). For group P, the increase for S3 was less than the increase for S2 and the increase for S3 was less than the increase for S1 and each was statistically significant ($p<0.05$, Figure 5).

Table 2. The average values of the salivary cortisol concentrations for both groups measured before and after each session

Sessions	Before / After	Group R (μg/dℓ) (mean±S.E.)	Group P (μg/dℓ) (mean±S.E.)
S1	0	0.223±0.029	0.272±0.066
	1	0.260±0.038	0.417±0.099
S2	0	0.354±0.077	0.375±0.065
	1	0.330±0.072	0.507±0.160
S3	0	0.370±0.066	0.434±0.079
	1	0.262±0.035	0.423±0.077

Sx (x=1, 2, 3), session order; 0, before the session; 1, after the session.

Table 3. The variation of the salivary cortisol concentrations for both groups measured before and after each session

Differences	Group R (μg/dℓ) (mean±S.E.)	Group P (μg/dℓ) (mean±S.E.)
S1	▲ 0.037±0.030	▲ 0.145±0.045
S2	▼ 0.024±0.030	▲ 0.132±0.102
S3	▼ 0.108±0.077	▼ 0.011±0.019

Differences: differences in salivary cortisol concentration between Sx−0 and Sx−1.

▲, increase; ▼, decrease.

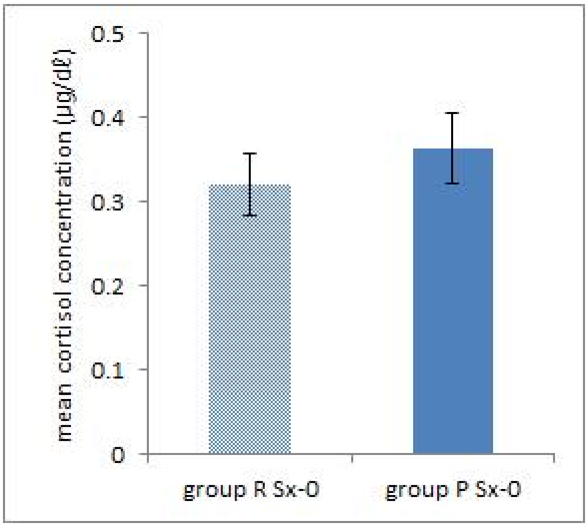

Figure 1. The average values of the Sx−0 salivary cortisol concentrations for both groups.

Sx−0 of two groups are not significantly different ($p>0.1$).
Sx (x=1, 2, 3), session order; 0, before the session.
Vertical lines represent standard errors of the means.

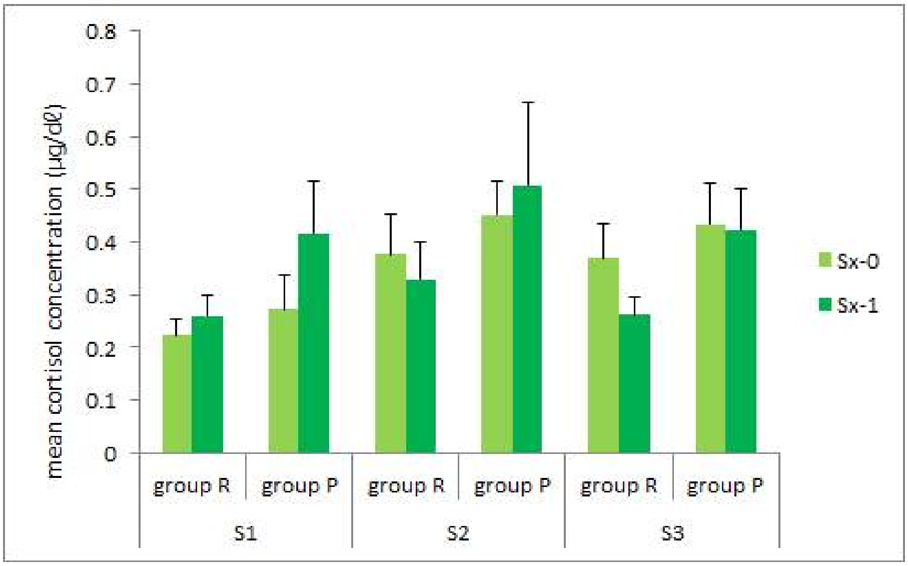

Figure 2. The average salivary cortisol concentration for S1, S2, S3 of each group.

Sx (x=1, 2, 3), session order; 0, before the session; 1, after the session. Vertical lines represent standard errors of the means.

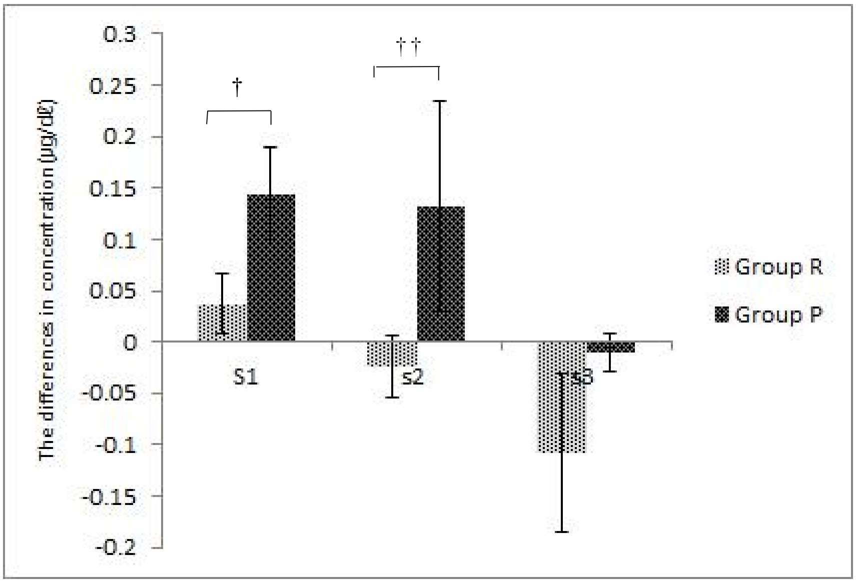

Figure 3. Change in salivary cortisol concentration between Sx−0 and Sx−1 for each group.

†: p<0.05, ††: p<0.1.

Vertical lines represent standard errors of the means.

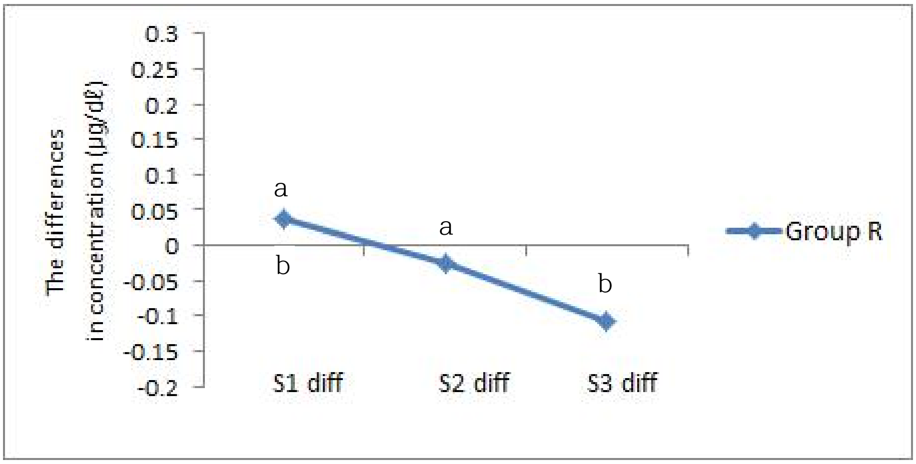

Figure 4. Decreasing trend in change of salivary cortisol concentration in each session for group R.

a: the two points marked a. The change in the salivary cortisol concentration for S2 decreased in comparison to S1.

b: the two points marked b. The change in the salivary cortisol concentration for S3 decreased in comparison to S1 (a, b: $p<0.1$).

Sx diff represents the difference between Sx-0 and Sx-1.

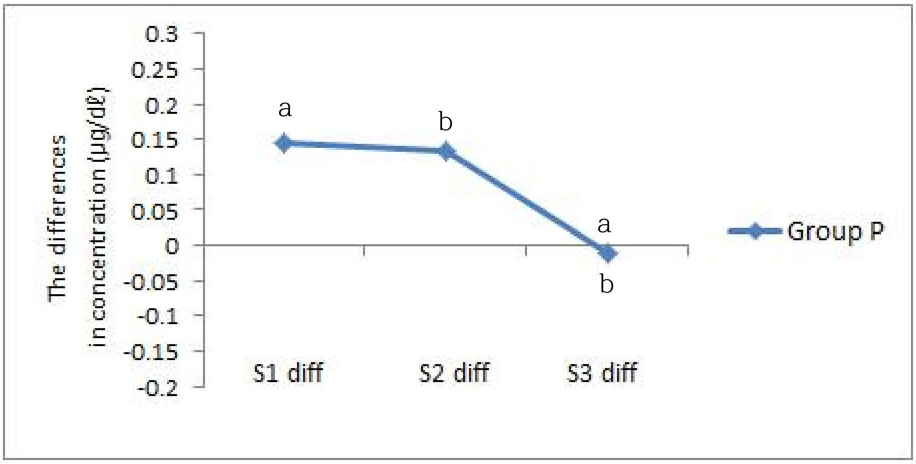

Figure 5. Decreasing trend in degree of change in salivary cortisol concentration in each session for group P.

a: the two points marked a. The change in the salivary cortisol concentration for S3 decreased in comparison to S1.

b: the two points marked b. The change in the salivary cortisol concentration for S3 decreased in comparison to S2 (a, b: $p<0.05$).

Sx diff represents the difference between Sx−0 and Sx−1.

4. Discussion

The objective of this experiment was to investigate the effect of reward-based and punishment-based training methods on the stress of dogs by analyzing the salivary cortisol concentration, which has recently become a popular non-invasive technique for research. To prove the hypothesis, the salivary cortisol concentration before and after each session was measured and compared. Nineteen dogs each received a total of 3 basic obedience training sessions. It was found that there was less of an increase in stress when using reward-based training methods than the punishment-based training methods for both S1 and S2. This result suggests that training a dog with reward-based training methods, rather than punishment-based training methods, induces less stress for the dog.

The experimental results of this study consistent with the results of previous studies that compare the two training methods. The reward-based training method has more advantages for various reasons, such not causing other behavioral problems (Blackwell *et al.*, 2008; Hiby *et al.*, 2004), being the most effective method for in rectifying existing behavioral issues, and having dogs voluntarily and actively participate in the training with a joyful attitude (Pryor, 1999; Yin, 2010). As a result, the dog's improved behavior makes them more attractive to people and increases the number of opportunities to positively interact with people leading to less stress experiences by the dog, since the dog is then able to create a predictable and controllable environment (Veissier and Boissy, 2007; Wells, 2004). In addition, as Serpell (1995) reported "the primary function of dogs has changed from an animal of utility to a companion animal." In modern society, 90% of pet dog owners consider dogs as companions and friends that they can spend a lifetime together with instead of working animals. They perceive the shared bond in the relationship as a form of reward given by the dog (Beaver, 2009; Marston and Bennett, 2003). Therefore, the reward-based training method has a positive impact on the relationship between the dog and its owner (Deldalle and Gaunet, 2014).

While there have been research results that reported punishment-based training methods give immediate effects, this method has more disadvantages overall, especially the provocation of aggressive behavior. The high probability of bringing about redirected aggression towards other dogs or people (Havebeke *et al.*, 2008a; Herron *et al.*, 2009; Hiby *et al.,* 2004; Rooney and Cowan, 2011) is problematic. Moreover, one study showed that working dogs trained using such methods exhibited low performance with regard to commands (Haverbeke *et al.*, 2008a). Dogs that received punishment-based method training, especially training involving physical punishments, tend to be cautious towards new people and environments and are less interactive with their caretaker. In other words, the training method and past activities affect the current behavior of dogs (Rooney and Cowan, 2011). Above all, using such aversive stimulation causes pain and damages the welfare of the dogs (Beerda *et al.*, 1997). The American College of Veterinary Behaviorists (2014) concluded, "punishment-based method has more disadvantages than advantages, due to the decreased well-being of the dogs and increased fear and anxiety."

Additionally, there is an interesting result obtained in this study. As the training progressed from S1 to S3, the salivary cortisol concentration variation between Sx-0 and Sx-1 showed a tendency to decrease in both groups. There was no statistical significance in the difference between the 3 session consecutively, from S1 to S2 and S3. However, the differences between two sessions was statistically significant. For group R, the decreasing trend in the change of cortisol concentration between S1 and S2, as well as S1 and S3, showed statistical significance ($p<0.1$). For group P, the decreasing trend of the cortisol variation between S1 and S3, as well as S2 and S3 showed statistical significance ($p<0.05$). Initially, the stress increase can be large because the dogs are placed in an unfamiliar situation when the training starts, but as the training nears completion, the dogs become more familiar and the situation becomes more predictable, resulting in the degree of change in stress. This result was with the results of the study by Haverbeke *et al.* (2008b). In this study, working dogs were given a challenge composed of 8 obedience training sessions and various stimuli and 2 sets of tasks separated by 20 days. The change in cortisol levels was measured to reveal that the cortisol levels initially increased, but the cortisol level the second time dropped below the level initially obtained and nearly equaled the base-line levels before the experiment. The results from the study by Henry and Stephens (1977) suggested that there is only stress when there is loss of control and a reduced predictability of what will happen. In other words, if the next events become predictable and the environment controllable, the dog will experience reduced stress levels. This process took place in this study as well over 3 training sessions.

For S1, both methods showed higher salivary cortisol concentrations for Sx-0 than for Sx-1; however, as the training progressed to S2 and S3, the salivary cortisol concentrations for Sx-1 decreased compared to Sx-0. For this result, it was thought that interacting with people and actively moving in the field relieved more stress than doing nothing in the unfamiliar training school other than being locked up in a kennel. In order to investigate whether human interaction can reduce the stress level of shelter dogs, Coppola *et al.* (2006) performed experiments on a group of shelter dogs that made contact with people and a group that did not. Only the group in contact with people underwent interaction sessions once a day for approximately 45 minutes over a few days. Activities such as reviewing basic obedience commands, going out to the outside kennels, playing, grooming, and petting were included. The no-contact group showed overall high cortisol concentrations compared to the contact group. On the day of the second session (third day in the shelter), the salivary cortisol concentration of the contact group decreased significantly ($p<0.05$); thus, it was concluded that for dogs placed in an unfamiliar environment, even a short period of interaction with people, including training, can act as a stress reliever for the dog. Haverbeke *et al.* (2008b) also reported that dogs might have found these challenges rather interesting and exciting compared to the barren kennel environment.

A review of studies on the salivary cortisol basal level of dogs was performed to confirm that the Sx−0 values of both groups in this study were the same. In previous studies, the salivary cortisol basal level of dogs was obtained, mainly through two methods. The first method uses the use of the value 0.156±0.061 ㎍/㎗ (range: 0.070−0.318 ㎍/㎗, CV=39%, Salimertics cortisol ELISA kit), obtained by Bennett and Hayssen (2010) from 45 regular pet dogs from households, as the basal salivary cortisol value in a wide range of papers. This value was obtained by the sampling and analysis of saliva from 48 dogs of various breeds and sex, followed by the removal of outliers.

Bennett and Hayssen (2010) states that this value can be used as the basal salivary cortisol value because the household pet dog is constantly in the same environment so it cannot be considered to involve stress factors. In their study, Hennessy *et al*. (1997) investigated dogs in shelter environments and state that it was not possible to find a basal level or standard value of cortisol concentration for shelter dogs since the shelter environment can not be considered 100% stress-free when compared to the household environment. Consequently, after the experiment, plasma cortisol level of 17 pet dogs living in a household environment was measured and used as the standard.

The second method employed by studies is to use the value measured before stimulation in each experimental environment as the basal salivary cortisol value. Bergamasco *et al*. (2010) used the salivary cortisol concentration value measured before the experimental session as the basal cortisol level (group A: 2.72±0.21 nmol/L, group B: 2.34±0.19 nmol/L) in order to study the effect of human interaction on shelter dogs. Beerda *et al*. (1998) used the average of the salivary cortisol concentration measured 15 and 30 minutes before stimulation (0.217 ㎍/dℓ), as the basal level.

In this study, in accordance with the latter method, the average of 3 cortisol concentrations (Sx-0, x=1, 2, 3) prior to the sessions was used as the basal level. As a result, the average cortisol concentration prior to the session for Groups R and P were 0.321±0.037 ㎍/㎗ and 0.363±0.041 ㎍/㎗, respectively (group R 95% confidence interval was 0.003~0.701 ㎍/㎗, group P 95% confidence interval was 0.003~0.769 ㎍/㎗). These values showed no statistical significance.

The basal level value used in this study was higher than the basal level of Bennett and Hayssen (2010). As can be inferred from the statement from Bennett and Hayssen (2010) that "dogs in the home environment may offer a means to measure 'basal cortisol' values not obtainable in 'chronic stress' environments such as kennels or shelters", the higher value of this study was thought to be a result of leaving their homes and find themselves in a stress inducing unfamiliar training school environment. The salivary cortisol concentration for dogs of various breeds living in shelters, kennels, and private homes were compared. The results show that the cortisol level of shelter dogs were higher than that of privately owned and kennel dogs (0.99, 0.58, 0.46 natural logarithm (ng/ml), respectively). These results are supported by the results of Sandri *et al.* (2015). The dogs participating in this study were those that had stayed at the training school for at least 5 days where the plasma cortisol numbers were 3 times that of household dogs up until the third day after arriving at the training school. This decreased to medium level values for the 4th to 9th days and then decreasing and plateaued on the 9th to 10th days. This result was consistent with the results from Hennessy *et al.*

(1997), where the cortisol level was still higher than that of regular household dogs. Rooney *et al.* (2007) studied the urinary cortisol levels of 31 labrador retrievers that moved to military training establishment kennels after being raised in regular households. The study revealed that the cortisol concentration remained higher than when at regular households for over 12 weeks after moving.

Blackwell *et al.* (2010) reported that "dogs appeared to become stressed when moved into unfamiliar kenneling". Most new environments involve unfamiliar environments where the dogs are separated from intimate people and other dogs and become with unacquainted people and dogs (Blackwell *et al.*, 2010). Dogs experience both social and spatial restrictions and the move to new kennels is a sufficient stress source. The move itself can be stressful. In addition, the typical kennel environment is an unpredictable external event where the environment is not controllable, social interaction with other dogs is limited, and noise levels are high. Hence, the cortisol levels for dogs moved into the kennel environment are higher than that of dogs in household environments (Coppola *et al.,* 2006; Hennessy *et al.*, 1997; Rooney *et al.*, 2007) The range of the measured cortisol levels for dogs that were moved into a new environment was wide. On average, it was at least double that of dogs in a domestic environment (Hennessy *et al.*, 1998; Rooney *et al.*, 2007; Stephen and Ledger, 2006).

The study by Hekman *et al.* (2012) investigated the relationship between the behavior and salivary cortisol levels of healthy dogs hospitalized for elective procedures and found cortisol results that differed from other reports. They determined the cause behind this difference was as follows: "1. The performance of the assay is laboratory specific, so the conclusion derived from the cortisol concentration should only be referenced for the specific laboratory setting (Briegel *et al.*, 2009), 2. Type 1 error resulting from small sample size, and 3. Hospitalization resulting in a change in the environment itself is a significant stress source. " Also, "using the same immune assay kit (Salimetrics, PA. USA) used in Belpedio *et al.* (2010), the salivary cortisol concentration range on the first day of moving to the shelter was 0.19−1.09 ㎍/dℓ" and the basal value of this study was consistent with this concentration range.

Meanwhile, the stress increase after the training in this study was found to be less than expected. This may be due to the fact that the subject dogs were middle and large−sized breeds and the Sx−1 cortisol concentration measurement time may have been too early. The cortisol concentration for large breed dogs were lower overall compared to that of small sized breeds (Sandri *et al.*, 2015), so the subject dogs of this study may have been more resistant against stress as they were all middle and large−sized breeds. With the exception of the middle−sized Welsh corgis, all the dogs of this study were large sized breed dogs (AKC, 2016). Beerda *et al.* (1998) measured the cortisol concentration of 10 dogs of various breeds, sex, and age after 10, 15, and 20 minutes of giving the dogs 6 aversive stimuli. The significant increase in the cortisol concentration started after 10 minutes and peaked at 20 minutes, followed by gradual a decrease, and returned to normal within an hour. In the study by Dreschel and Granger (2005) of thunderstorm phobic dogs, the cortisol concentration was measured before being exposed to a simulated thunderstorm, 20 minutes after exposure, and 40 minutes after exposure. The study revealed that the highest cortisol concentration was measured 20 minutes after exposure and began to decrease 40 minutes after exposure.

In this study, the Sx-1 measurement time was set to 10 minutes after taking into consideration the study results that showed that short and repetitious training sessions no longer than 10 minutes are most effective (Pryor, 1999). The study result showed that handling does not affect the cortisol value for up to 4 minutes (Kobelt *et al.*, 2003). The influence of the handling during the Sx-0 sampling on the Sx-1 value. If the Sx-1 measurement was performed 15-20 minutes after, similar to some other studies (Hováth, *et al.*, 2007; Vincent and Michell, 1992), the cortisol increase could have been slightly greater.

In this study, there were variations in the subjects' sex, breed, size, and age; but, according to previous literature, no significant correlations between sex or age and salivary cortisol concentration have been reported (Coppola *et al.*, 2006; Hennesy *et al.*, 1997; Stephen and Ledger, 2006). Moreover, no significant differences in the cortisol concentration were found according to the breed (Coppola *et al.*, 2006). Bennett and Hayssen (2010) also reported no observed relationship between the salivary cortisol levels and age, sex, weight, and neutered status, so it was determined that the subject dogs and the experiment results of this study were reliable. Since a training school that employs both training methods could not be found, the subject dogs could not be trained at the same training school or by the same trainer. The study of Deladalle and Gauner (2014) used as the reference for where the experimentation carried out in different training facilities. Similarly, they also compared two training methods.

In the experiment of this study, behavioral analysis was not carried out; however, simple observation showed that most of the dogs for both methods properly responded to both or one of the hand or vocal signals for sit, lie down, and wait when the third training session completed. Moreover, the behavior or attitude of the dogs participating in the training session were completely different between the two groups rather than differences in the training accomplishments. If the behavioral characteristics of the dogs for each test group were described using words, group R dogs were motivated, proactive, quick, cheerful, maintained eye contact, excited, and tail wagging, Group P dogs were passive, slow, tense, showed stress related behavior, had low postures, and evaded eye contact.

Recently, various studies have been published reporting the scientific effects and humane aspects of employing reward-based training methods or positive reinforcement when training (educating) dogs, resulting in the gradual adoption of positive reinforcement as the major training method of organizations that train professional detector dogs (Hiby *et al.*, 2004). However, negative reinforcement or punishments perceived as the traditional method are mainly being used as the training method for working dogs as well as regular pet dogs (Herron *et al.*, 2009; Hiby *et al.*, 2004). Such punishment-based training methods are not only used by training schools and facilities throughout the country but also propagated by their appearances on television programs (Yin *et al.*, 2008). The survey conducted by Blackwell *et al.* (2008) showed that 88% of dog breeders receive various training with the dogs where only 16% of them used +R while the rest used a mix of the methods. 72% of methods used included +P. Moreover, the current reality is due to the misconception that using the convenient forceful method is not bad since training, regardless of it being reward-based or punishment-based, must inevitably cause some stress for the dog due to the outdated perception that the dog owner must be dominant over the dog and aggressive towards the dog like that of an ‘alpha dog’.

In modern society, dogs that have no need to work can receive psychological and physical stimuli through training as a means to exert their drive. Furthermore, basic training is a necessary element in rectifying problematic behavior. Yin *et al.* (2008) reported that positive reinforcement is the best behavior correction method along with counter conditioning. Small and significant behavioral problems can be corrected through basic training alone, and learning outcomes can be amplified when using positive reinforcement based training. Training is necessary to control dogs for the purpose of safety, to prevent the harming of other people, to enhance the intimacy between the dog and its owner, and to foster the proper socialization, welfare, and enrichment of pet dogs (Dunbar, 2004; Yin, 2010).

Up to now, studies on dog training were focused on military, police or laboratory dogs as they were relatively easier to bring together and are uniform as experiment subjects. Such studies are quite dissociated with the normal companion dog training that modern society needs. There is the limitation of difficulty in getting the agreement of private training facilities to study in a non-laboratory environment since such facilities are hesitant to reveal their training expertise. It is not easy to have the same experiment subjects and conditions. Research comparing the two training methods has only begun recently, so reference data is not abundant, but this is a time where the importance of regular dog training is emphasized. This on-site study is expected to contribute practically.

The objective of this study was to verify that the recently spotlighted reward-based training method causes less stress to dogs in comparison to the typically used forceful training methods for dogs that need basic training. This is to reduce abandonment, disownment, and administration of euthanasia due to behavioral problems that compromise the welfare of dogs, as well as cause serious social issues. The result of this study showed that the reward-based training method was less stressful for the dogs compared to punishment-based training method. This research is expected to present the training method that is more beneficial towards the welfare of dogs. It also comes at a time when animal welfare and ethics are important considerations. Active domestic studies using the non-invasive measurement of salivary cortisol concentration that is less stressful on animals would conform to the 3Rs of experiment animal ethics (Replacement, Refinement and Reduction). The results of this study are expected to contribute to the replacement of deeply ingrained and punishment-based training methods with reward-based training methods.

5. Conclusion

This study, taking into consideration the increasing importance of socialization and basic obedience training of regular canines, measured the salivary cortisol levels of dogs in order to investigate the effect of the training method on the stress experienced by dogs. The changes in salivary cortisol level measured before and after 3 training sessions revealed that the cortisol increase was smaller for the reward-based method where S1 and S2 showed statistical significance of $p<0.05$ and $p<0.1$, respectively. Furthermore, both groups underwent repeated sessions, showing decreases in the degree of cortisol concentration increase as the training progressed. Group R showed statistical significance between S1 and S2 as well as S1 and S3 ($p<0.1$) while group P showed statistical significance between S2 and S3 as well as S1 and S3 ($p<0.05$).

In the reality of today where many dog owners and trainers still employ the traditional punishment-based methods, the results of this study are expected to provide insight into which training method is more beneficial for animal ethics and towards the welfare of dogs.

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국문초록

개에서 타액 코티솔 측정을 통한 훈련방법에 따른 스트레스 유발 수준의 비교

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이 연구의 목적은 개의 훈련 방법 중 '보상 기반 훈련법(reward-based training method)'과 '처벌 기반 훈련법(punishment-based training method)'이 개에게 유발하는 스트레스에 어떤 차이가 있는지를 타액 코티졸 농도 측정을 통해 알아보는 것이다. 개가 인간 사회에서 신체적, 정신적으로 건강하게 살아가기 위해서는 사회화 훈련 및 기초 복종 훈련이 반드시 필요하다. 여러 가지 훈련법이 혼재하는 가운데, 이 실험에서

는 훈련방법에 따라 개의 스트레스 변화량에 어떤 차이가 있는지 알아보기 위해, 보상 기반 훈련법(group R, n=10)과 처벌 기반 훈련법(group P, n=9)을 사용하는 훈련소의 개들을 각각 1, 2, 3차(Sx, x=1, 2, 3)에 거쳐 기초 복종 훈련에 속하는 '앉아', '엎드려', '기다려'를 가르치면서 훈련 session 전(Sx-0)과 후(Sx-1) 간의 타액 코티솔 농도 변화량을 비교 측정해 보았다. Swab을 개의 볼주머니에 1분간 물고 있게 해서 타액 샘플을 모았으며, 분석은 고감도 코티솔 농도 측정 키트를 사용해 이뤄졌고 각 타액 샘플의 평균 흡광도(O.D.)를 측정한 뒤 산출된 타액 코티솔 농도를 ㎍/dℓ로 변환했다. 훈련 session 차수 별로 각 실험군의 세션 전·후 간의 타액 코티솔 농도 변화량을 비교해 본 결과, S1에서의 실험군 R과 실험군 P의 세션 전·후 간의 타액 코티솔 농도 변화량은 각각 0.037±0.030㎍/dℓ와 0.145±0.045㎍/dℓ로 증가해 실험군 R의 타액 코티솔 농도 증가량이 더 적었고 통계적 유의성이 나타났다($p<0.05$). S2에서도 실험군 R과 실험군 P의 세션 전·후 간의 타액 코티솔 농도 변화량은 각각 -0.024±0.030 ㎍/dℓ와 0.132±0.102 ㎍/dℓ으로 실험군 R의 타액 코티솔 농도 증가량이 더 적었으며 통계적 유의성이 나타났다 ($p<0.1$). S3에서는 실험군 R과 실험군 P의 세션 전·후 간의 타액 코티솔 농도 변화량은 각각 -0.108±0.077㎍/dℓ와 -0.011±0.019㎍/dℓ로 실험군 R의 타액 코티솔 농도 증가량이 더 적은 경향성을 보였지만 통계적 유의성은 없었다. 한편, 두 그룹 모두 session이 반복되어 학습이 완성될수록 코티솔 증가량이 감소한다는 추가적인 결과도 얻었는데, group R의 경우는 S1과 S2, S1과 S3 간에 각각 통계적 유의성이 나타났고($p<0.1$) group P의 경우 S2와 S3, S1과 S3간에 각각 통계적 유의성이 나타났다($p<0.05$). 본 실험을 통해 전반적으로 보상 기반 훈련법이 처벌 기반 훈련법에 비해 훈련 전·후 간

의 스트레스 증가량이 더 적다는 것을 알 수 있었다. 따라서 이 연구 결과는 보상 기반 훈련법을 사용하는 것이 동물 윤리 및 개의 복지 측면에서 볼 때 더 이롭다는 것을 시사하며 관련 후속 연구에 기여할 것으로 생각된다.

주요어: 개, 타액 코티솔, 훈련방법, 스트레스
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